PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT	:	NG, et al.
SERIAL NO	:	10/004,968
FILED	:	December 25, 2001
TITLE	:	METHODS AND COMPOSITIONS FOR HIGHLY EFFICIENT PRODUCTION OF HETEROLOGOUS PROTEINS IN YEAST
Grp./A.U.	:	1636
Examiner	:	VOGEL, Nancy S.
Conf. No.	:	7842
Docket No.	:	P05424US01

DECLARATION OF DAVIS T. W. NG
UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Davis T. W. Ng, hereby declare:

1. I am an Assistant Professor of biochemistry and molecular biology at Pennsylvania State University, and a co-inventor of the above-identified application.

2. I have a PhD in molecular and cell biology from Northwestern University. My research focuses on mechanisms that mediate and regulate the biosynthesis of secretory pathway proteins using the model organism the budding yeast *Saccharomyces cerevisiae*.

3. A copy of my CV is attached herewith (Exhibit A, 3 pages).

4. I have read the Office Action dated January 29, 2004 and am familiar with the references cited by the Examiner.

5. This Declaration is submitted herein to demonstrate that Applicants' methods are distinguishably patentable over the cited references.

6. As disclosed and evidenced herein, I have demonstrated the folding of KGFP, a reporter construct, which is disclosed in the above-identified application, is enhanced with a PMT2 mutant and exhibits even greater enhancement with a PMT1\PMT2 double mutant yeast cell. Since KGFP is a fluorescent marker, folding can be monitored by changes in emission activity. Exhibit B demonstrates that a cell wherein the PMT2 gene has been inhibited has a greater relative GFP fluorescence that a PMT1 mutant, which is evidence that following the method of Tanner is not inherent to Applicants' claimed method of facilitating or promoting proper folding of heterologous proteins by inhibition of the PMT1 gene. Moreover, the greatest effects of folding and assembly came with a PMT1\PMT2 double mutant (CBY1), as shown in Exhibit B. This demonstrates that folding and assembly can be disrupted in wild-type yeast cells and improved in a PMT2 mutant cell or a PMT1\PMT2 double mutant cell. As shown from Exhibit B, PMT1 emitted less fluorescence, thereby indicating it had little benefits to improving folding over both PMT2 or a PMT1\PMT2 double mutant yeast cell.

7. Although the improvement of KGFP folding was dramatic, I sought to extend the generality of the invention to other proteins. A large class of human therapeutic molecules is humanized monoclonal antibodies. To determine whether the invention would provide benefits for their synthesis, clones encoding the λ-light and γ-heavy chains of human IgG2 were engineered for yeast (*Saccharomyces cerevisiae*) expression. The mammalian signal sequences were replaced at the nucleotide level with those from the *SUC2* (encoding invertase) or *KAR2* genes (data from the *SUC2* fusions will be presented in this section). Intron sequences were deleted precisely and the resulting genes placed under the control of the yeast *TDH3* promoter

and *ACT1* terminator. Figure 1 (below) shows the coding sequences of the invertase/λ-light chain fusion (designated 'ILC') and of the invertase/γ-heavy chains (designated 'IHC'). The genes were inserted into yeast centromeric vectors to generate pGAU-IHC (*URA3*-marked) and pGLA-ILC (*LEU2*-marked). The pair of plasmids were simultaneously transformed into wild-type (W303, *MAT***a**), CBY237 (*MAT***a**, *pmt2::KANX*), and CBY262 (*MAT***a**, *pmt1::KANX, pmt2::KANX*) strains for the expression of both proteins. Strains were grown on synthetic complete (SC) media lacking uracil and leucine to select for maintenance of both plasmids.

The heavy chain has a single site for N-linked glycosylation while the light chain is non-glycosylated. To analyze the glycosylation of the heavy chain, wild type, Δ*pmt2*, and Δ*pmt1*/Δ*pmt2* strains expressing anti-IL8 IgG were pulse-labeled for 10' with [^{35}S]-methionine/cysteine and chased with cold amino acids for 0, 15, and 30 minutes. Labeled anti-IL8 IgG was immunoprecipitated using anti-human IgG antibody. Immunoprecipitated proteins were either mock treated or digested with endoglycosidase H to remove N-linked carbohydrates. Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and visualized by autoradiography. As shown in Figure 2, anti-IL8 heavy chain resolves into three distinct bands following the pulse in both wild type and Δ*pmt2* cells (compare lanes 1 and 4).

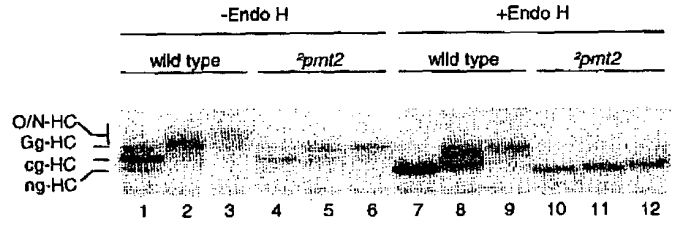

Figure 2. Maturation of anti-IL8γ-chain. Wild type and Δ*pmt2* cells expressing anti-IL8 antibody were pulse-labeled for 10 min with 35S-met/cys and chased for times indicated. Labeled IgG was immunoprecipitated using rabbit anti-human IgG, separated on a 10% SDS-PAG and visualized by autoradiography. Immunoprecipitated protein was either mock or Endo H treated before eletrophoresis. The positions of non-glysylated (ng-HC), core glycosylated (cg-HC), Golgi-modified (Gg-HC), and O- and N-linked modified (O/N-HC) γ heavy chain are indicated.

The identity of the fastest migrating band (ng-HC) as the non-glycosylated form was determined since it co-migrates with deglycosylated heavy chain (compare lanes 1 and 4 with lanes 7 and 10). The next fastest band is the core glycosylated band comprised of a single N-linked carbohydrate (cg-HC). That form chases to the Gg-HC form due to the extension of the N-linked moiety in the Golgi apparatus (lanes 2 and 5). Interestingly, the heavy chain produced in wild type cells is differently modified than that expressed in Δ*pmt2* cells. In Δ*pmt2* cells, the protein is modified only to the Gg-HC form while, in wild type cells, the protein exhibits a further decrease in mobility indicating that it is modified by O-mannosylation. The identity of this form was confirmed after removal of N-linked carbohydrates by endoglycosidase H. As shown in lanes 8 and 9, the mobility difference persists after removal of N-linked sugars. By contrast, heavy chain produced by Δ*pmt2* cells and deglycosylated generates a band that co-migrates with unglycosylated heavy chain (lanes 10-12). This confirms that heavy chain produced in wild type cells is significantly modified by O-mannosylation. This is an important discovery since O-mannosylation of proteins does not occur in mammalian cells and proteins modified in this way would likely be immunogenic and affect the activity of the expressed protein. Disruption of the *PMT1* and *PMT2* genes prevents this undesirable modification.

To analyze the assembly of the whole antibody, wild type and Δ*pmt2* cells expressing anti-IL8 antibody were pulse-labeled for 10 min, chase for 0, 15, and 30 min, IgG immunoprecipitated and resolved by non-reducing SDS-PAGE. In assembled antibody, two light chains assemble with two heavy chains that are joined by covalent disulfide bonds. Assembly can be assayed on non-reducing gels as the tetramer migrates at about 160 kD (Figure 3, HC_2LC_2). As shown in Figure 3, anti-IL8 antibody assembles correctly into tetramers in both wild type and Δ*pmt2* cells. Importantly, the assembled antibody from wild type cells shows the time-dependent

decrease in mobility observed on the reducing gel (Figure 2). That this change is due to O-mannosylation was confirmed by the absence of a mobility shift in Δ*pmt2* cells (Figure 3, lanes 3-6).

To analyze the efficiency of assembly, wild type, Δ*pmt2*, and Δ*pmt1*/Δ*pmt2* cells expressing anti-IL8 antibody was pulse-labeled for 10 min, chased for 15 min, immunoprecipitated and analyzed as performed in Figure 3. Relative amounts of monomeric heavy chain and tetramer were quantified by phosphorimager analysis and plotted as percent tetramer. As shown in Figure 4, 18.6% of heavy chain in wild type cells assembled into tetramer at the 15 min time point compared with 27.4% and 28.9% of heavy chain in Δ*pmt2* and Δ*pmt1*/Δ*pmt2* cells, respectively. Although not as dramatic as for KGFP, the increase in folding

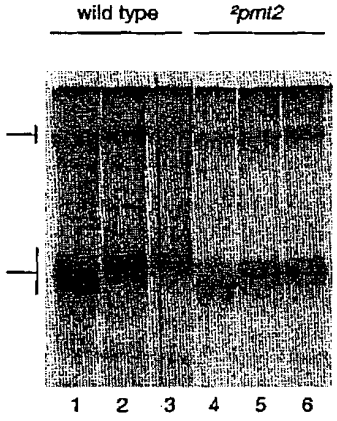

Figure 3. Assembly of tetrameric anti-IL8 IgG. Wild type and ²pmt2 cells were pulse-labeled with [35S]methionine/cysteine for 10 min and chased for the times indicated and immunoprecipitated with anti-human IgG. Proteins were resolved by non-reducing SDS-PAGE. The positions of the tetramer (HC_2LC_2) and heavy chain monomer (HC_1) are indicated.

efficiency for tetrameric antibody was significant at 47% and 55% increases in the Δ*pmt2* and Δ*pmt1*/Δ*pmt2* cells, respectively over wild type. This provides clear evidence that the invention is beneficial for increasing the folding/assembly of a second molecule that is more complex than the green fluorescent protein. Equally important, the prevention of the aberrant O-mannosylation is likely to be critical for preventing undesirable effects such as immunogenicity and loss of activity. Taken together, the data show that the invention has general applications for improving

the folding and expression of heterologous proteins and that aberrant O-mannosylation is a common occurrence that can be prevented using specifically engineered strains of yeast.

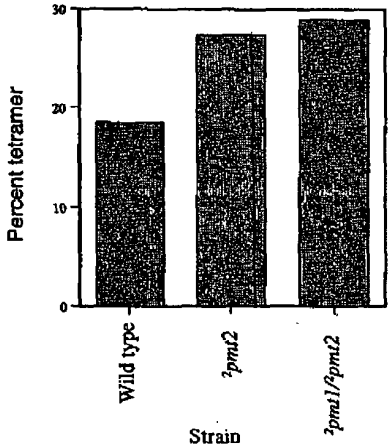

Figure 4. Assembly of anti-IL8 antibodies. Wild type $^{2}pmt2$, and $^{s}pmt1/^{2}pmt2$ cells expressing anti-IL8 antibody were pulse-labeled for 10 min with 35S-methionine/cysteine and chased for 15 min. Proteins immunoprecipitated with anti-human IgG resolved by non-reducing SDS-PAGE and tetramer and monomer species quantified by phosphorimager analysis (Molecular Dynamics Typhoon system). The data are plotted as the percent tetramer of total.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Dated this 27th day of May, 2004

Davis T. W. Ng